EXHIBIT A

20.8.96

Lösung für Pre-Hybridisierung / Hybridisierung

[illegible] 0.5 M (P) : Phosphatpuffer
7 % SDS
0.5 mM EDTA

→ erhitzen bis klar

Blots : 8 × GnT I : 3 × Northern
5 × Southern
3 × GnT II : 3 × Southern

GnT I :

1.) Northern total RNA 8 tissues (5.1.96) : •
2.) —"— (9.2.96) : •• (kühlbox aufgetaut / Heidelberg)
3.) Northern poly(A)+ 6 tissues (8.1.96) •••
Southern:
4.) RT-PCR p16/22 t.RNA 8 tissues (14.6.96) : ••••
5.) RT-PCR p20/21 t.RNA 8 —"— RsaI red (8.8.96) : •••••
6.) RT-PCR p20/21 t.RNA 4 small tissues RsaI red (9.8.96) ••••••
7.) RT-PCR p42/22 t. RNA 8 tissues PCR v. 14.8.96 (19.8.96) : •••••••
(5 U AMV RT / [illegible])
8.) RT-PCR p42/22 t.RNA 8 tissues PCR v. 15.8.96 (19.8.96) ••••••••
(10 U AMV RT)

(gekennzeichnet mit Nadel ein Loch am Rand gestochen)

GnT II : [illegible]

9.) RT-PCR p31/32 t.RNA 8 tissues (14.6.96) : •••••••••
10.) RT-PCR p31/32 t.RNA 8 tissues NdeI red (14.8.96) : ••••••••••
11.) —"— (19.8.96) : •••••••••••

alle Southern Blot Membranen "cross-linked"
→ auf ein Papier gelegt, aktive Seite oben
mit Frischhaltefolie bedeckt → cross-linking im
Hoefer Scientific [illegible]

EXHIBIT B

Table 2.10.3 High-Salt Solutions Used in Hybridization Analysis

Stock solution	Composition
20×SSC	3.0 M NaCl/0.3 M trisodium citrate
20×SSPE[a]	3.6 M NaCl/0.2 M NaH_2PO_4/0.02 M EDTA, pH 7.7
[illegible]	1 M $NaHPO_4$, pH 7.2[c]

[a]SSC may be replaced with the same concentration of SSPE in all protocols.

[b]Prehybridize and hybridize with 0.5 M $NaHPO_4$ (pH 7.2)/1 mM EDTA/7% SDS [or 50% formamide/0.25 M $NaHPO_4$ (pH 7.2)/0.25 M NaCl/1 mM EDTA/7% SDS]; perform moderate-stringency wash in 40 mM $NaHPO_4$ (pH 7.2)/1 mM EDTA/5% SDS; perform high-stringency wash in 40 mM $NaHPO_4$ (pH 7.2)/1 mM EDTA/1% SDS.

[c]Dissolve 134 g $Na_2HPO_4 \cdot 7H_2O$ in 1 liter water, then add 4 ml 85% H_3PO_4. The resulting solution is 1 M Na^+, pH 7.2.

Designing washes for heterologous hybridization. Calculations of T_m become more critical if heterologous probing is being attempted. If the aim is to identify sequences that are merely related, not identical, to the probe (e.g., members of a multigene family, or a similar gene in a second organism), then it is useful to have an idea of the degree of mismatching that will be tolerated by a "moderate-" or "low-" stringency wash. The best way to approach this is to first establish the lowest temperature at which only homologous hybridization occurs with a particular SSC concentration. Then assume that 1% mismatching results in a 1°C decrease in the T_m (Bonner et al., 1973) and reduce the temperature of the final wash accordingly (for example, if sequences with ≥90% similarity with the probe are being sought, decrease the final wash temperature by 10°C). If the desired degree of mismatching results in a wash temperature of <45°C, then it is best to increase the SSC concentration so that a higher temperature can be used. Doubling the SSC concentration results in a ~17°C increase in T_m, so washes at 45°C in 0.1× SSC and 62°C in 0.2× SSC are roughly equivalent. Note that in these extreme cases it may also be necessary to reduce the hybridization temperature to as low as 45°C (aqueous solution) or 32°C (formamide solution).

This approach sometimes works extremely well (as shown when the heterologous targets are eventually sequenced), but the assumption that a 1% degree of mismatching reduces the T_m of a heteroduplex by 1°C is very approximate. Base composition and mismatch distribution influence the actual change in T_m, which can be anything between 0.5° and 1.5°C per 1% mismatch (Hyman et al., 1973). Unfortunately trial and error is the only alternative to the "rational" approach described here.

Other parameters relevant to hybridization analysis

Length of prehybridization and hybridization incubations. The protocols recommend prehybridization for 3 hr with nitrocellulose and 15 min for nylon membranes. Inadequate prehybridization can lead to high backgrounds, so these times should not be reduced. They can, however, be extended without problem.

Hybridizations are usually carried out overnight. This is a rather sloppy aspect of the procedure, because time can have an important influence on the result, especially if, as described above, an excess amount of a single-stranded probe is being used. The difficulties in assigning values to the parameters needed to calculate optimum hybridization time has led to the standard "overnight" incubation, which in fact is suitable for most purposes. The exception is when hybridization is being taken to its limits, for instance in detection of single-copy sequences in human DNA, when longer hybridization times (up to 24 hr) may improve sensitivity if a single-stranded probe is being used. Note that this does not apply to double-stranded probes, as gradual reannealing results in only minimal amounts of a double-stranded probe being free to hybridize after ~8 hr of incubation.

Formamide hybridization buffers. Formamide destabilizes nucleic acid duplexes, reducing the T_m by an average of 0.6°C per 1% formamide for a DNA-DNA hybrid (Meinkoth and Wahl, 1984) and rather less for a DNA-RNA hybrid (Casey and Davidson, 1977; Kafatos et al., 1979). It can be used at 50% concentration in the hybridization solution, reducing the T_m so that the incubation can be carried out at a lower temperature than needed with an aqueous solution. Originally formamide was used with nitrocellulose membranes as a means of prolonging their lifetime, as the

lower hybridization temperature results in less removal of target DNA from the matrix. More recently formamide has found a second use in reduction of heterologous background hybridization with RNA probes. RNA-DNA hybrids are relatively strong, and heterologous duplexes remain stable even at high temperatures. The destabilizing effect of formamide is therefore utilized to maximize the discrimination between homologous and heterologous hybridization with RNA probes.

Formamide probably confers no major advantage on DNA-DNA hybridization with a nylon membrane. In fact it introduces two problems, the hazardous nature of the chemical itself, and an apparent reduction in hybridization rate. The latter point is controversial (Hinton, 1977), but for equivalent sensitivity a formamide hybridization reaction usually has to incubate for longer than an aqueous one.

Alternatives to SSC. Although SSC has been used in hybridization solutions for many years, there is nothing sacrosanct about the formulation, and other salt solutions can be employed (Table 2.10.3). There is little to choose between these alternatives. SSPE and phosphate solutions have a greater buffering power and may confer an advantage in formamide hybridization solutions. Alternatively, the buffering power of SSC can be increased by adding 0.3% (w/v) tetrasodium pyrophosphate.

Probe length. Probe length has a major influence on the rate of duplex formation in solution hybridization (Wetmur and Davidson, 1968), but the effect is less marked when the target DNA is immobilized. In membrane hybridization a more important factor is the specificity of the probe. The probe should never be too long (>1000 bp), as this increases the chance of heterologous duplexes remaining stable during a high-stringency wash. Neither should the probe contain extensive vector sequences, as these can hybridize to their own target sites, wrecking the specificity of the experiment.

Mechanics of hybridization. Traditionally hybridization has been carried out in plastic bags. This technique is messy, radiochemical spills being almost unavoidable, and can lead to detrimental contact effects if too many membranes are hybridized in a single bag. Hybridization incubators are now available from a number of companies and are recommended as a distinct advance over the plastic bag technology. Rotation of the hybridization tube results in excellent mixing, reducing hot spots caused by bubbles and dust and leading to very evenly hybridized membranes. Good-quality results are possible even when ten or more minigel Southerns are hybridized in a single 8.5 × 3.0–inch tube.

If bags are used, they should be of stiff plastic to prevent the sides collapsing on to the membrane, which will lead to high background. The volume of hybridization solution should be sufficient to fill the bag, and no more than two membranes should be hybridized in each bag.

Troubleshooting

Problems in blotting and hybridization reveal themselves when the autoradiograph is developed. A guide to the commonest problems and how to solve them is given in Table 2.10.4 (based on Dyson, 1991).

A particularly troublesome problem is high background signal across the entire membrane. This is due to the probe attaching to nucleic acid binding sites on the membrane surface, the same sites that bind DNA during the blotting procedure. Prehybridization/hybridization solutions contain reagents that block these sites and hence reduce background hybridization. The most popular blocking agent is Denhardt solution, which contains three polymeric compounds (Ficoll, polyvinylpyrrolidone, and BSA) that compete with nucleic acids for the membrane-binding sites. The formulations used in the basic and alternate protocols also include denatured salmon sperm DNA (any complex DNA that is nonhomologous with the target is acceptable) which also competes with the probe for the membrane sites. Blocking agents are included in the prehybridization solution to give them a head start over the probe. With a nylon membrane, the blocking agents may have to be left out of the hybridization solution, as they can interfere with the probe-target interaction. When the membranes are washed, the Denhardt solution and salmon sperm DNA are replaced with SDS, which acts as a blocking agent at concentrations ≥1%.

Other blocking agents can also be used (Table 2.10.5). With DNA blots, the main alternatives to Denhardt are heparin (Singh and Jones, 1984) and milk powder (BLOTTO; Johnson et al., 1984), although Denhardt is generally more effective, at least with nylon membranes. Note that BLOTTO contains RNases and so can be used only in DNA-DNA hybridizations. With an RNA probe, denatured salmon sperm DNA is sometimes replaced by 100 µg/ml yeast tRNA, which has the advantage that it does not need to be sheared before

EXHIBIT C

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Purification, cDNA Cloning, and Expression of GDP-L-Fuc:Asn-linked GlcNAc α1,3-Fucosyltransferase from Mung Beans*

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Substitution of the asparagine-linked GlcNAc by α1,3-linked fucose is a widespread feature of plant as well as of insect glycoproteins, which renders the *N*-glycan immunogenic. We have purified from mung bean seedlings the GDP-L-Fuc:Asn-linked GlcNAc α1,3-fucosyltransferase (core α1,3-fucosyltransferase) that is responsible for the synthesis of this linkage. The major isoform had an apparent mass of 54 kDa and isoelectric points ranging from 6.8 to 8.2. From that protein, four tryptic peptides were isolated and sequenced. Based on an approach involving reverse transcriptase-polymerase chain reaction with degenerate primers and rapid amplification of cDNA ends, core α1,3-fucosyltransferase cDNA was cloned from mung bean mRNA. The 2200-base pair cDNA contained an open reading frame of 1530 base pairs that encoded a 510-amino acid protein with a predicted molecular mass of 56.8 kDa. Analysis of cDNA derived from genomic DNA revealed the presence of three introns within the open reading frame. Remarkably, from the four exons, only exon II exhibited significant homology to animal and bacterial α1,3/4-fucosyltransferases which, though, are responsible for the biosynthesis of Lewis determinants. The recombinant fucosyltransferase was expressed in Sf21 insect cells using a baculovirus vector. The enzyme acted on glycopeptides having the glycan structures GlcNAcβ1–2Manα1–3(GlcNAcβ1–2Manα1–6)Manβ1–4GlcNAcβ1–4GlcNAcβ1-Asn, GlcNAcβ1–2Manα1–3(GlcNAcβ1–2Manα1–6)Manβ1–4GlcNAcβ1–4(Fucα1–6)GlcNAcβ1-Asn, and GlcNAcβ1–2Manα1–3[Manα1–3(Manα1–6)Manα1–6]Manβ1–4GlcNAcβ1–4GlcNAcβ1-Asn but not on, *e.g.* *N*-acetyllactosamine. The structure of the core α1,3-fucosylated product was verified by high performance liquid chromatography of the pyridylaminated glycan and by its insensitivity to *N*-glycosidase F as revealed by matrix-assisted laser desorption/ionization time of flight mass spectrometry.

The most characteristic features of asparagine-linked oligosaccharides from plants are the substitution of the core pentasaccharide by xylose and α1,3-linked fucose (1, 2). The resulting heptasaccharide "MMXF^{3}" (Fig. 1) very often constitutes the main oligosaccharide species on a plant glycoprotein (3, 4). According to their biosynthesis, these structures are classified as complex-type *N*-glycans, even though the terms paucimannosidic or truncated *N*-glycans appear to be more justified. The α-mannosyl residues may, however, be substituted by GlcNAc and these GlcNAc residues may be further decorated by galactose and fucose to form the same structure as the human Lewis a epitope (Fig. 1) (5, 6).

The antigenicity of "paucimannosidic" plant *N*-glycans is well documented (7–11). Since both xylose and core α1,3-fucose are not seen in mammalian glycoproteins they may form the key component of epitopes for carbohydrate-reactive antibodies (9, 10, 12). There is, however, evidence that the α1,3-linked fucosyl residue is the predominant antibody binding structural element (3, 8, 11, 13). Due to the ubiquitous occurrence of such paucimannosidic *N*-glycans throughout the plant kingdom, they are responsible for the frequently observed cross-reactivity of antibodies raised against plant glycoproteins and are therefore termed "cross-reactive carbohydrate determinants" (12, 14, 15). Anti-cross-reactive carbohydrate determinants antibodies of the IgE class have been found in sera of many allergic patients (8, 11, 13, 14, 16, 17). While the clinical role of cross-reactive carbohydrate determinants remains controversial, they are suspected to obscure (at least *in vitro*) allergy diagnosis. Anti-cross-reactive carbohydrate determinants antibodies will also react with many insect glycoproteins such as honeybee venom phospholipase A_2 or neuronal membrane glycoproteins from insect embryos because insects, like plants, are capable of synthesizing the core α1,3-fucose epitope (3, 11–13, 18, 19).

In contrast to the blood group-related fucosyltransferases which act on the nonreducing terminus of *N*-glycans, *O*-glycans, or glycolipids (20), core fucosyltransferases have received little attention. Only recently, the molecular cloning of GDP-L-Fuc[1]:Asn-linked GlcNAc α1,6-fucosyltransferase (core α1,6-fucosyltransferase, Fuc-T C6, Fuc-T VIII) from porcine brain and from human gastric cancer cells has been reported (21, 22). As regards core α1,3-fucosyltransferase (Fuc-T C3), a first charac-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y18529.

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[1] The abbreviations used are: Fuc, L-Fucose; Fuc-T C3, GDP-L-Fuc:Asn-linked GlcNAc α1,3-fucosyltransferase (core α1,3-fucosyltransferase); Fuc-T C6, GDP-L-Fuc:Asn-linked GlcNAc α1,6-fucosyltransferase (core α1,6-fucosyltransferase); GnGn, GnGnF^{3}, and GnGnF^{6}, *N*-glycans, for structures, see Fig. 1; HPLC, high performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; GnGn, GnGnF^{6}, GnGnF^{3}F^{6}, GnGnF^{3}, MMF^{3}, GalGal, GalGnF^{3}, GnGal, MM, M5Gn, and M5GnF^{3}, *N*-glycans, for structures see Fig. 1, PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; Mes, 2-(*N*-morpholino)ethanesulfonic acid; bp, base pair(s).

This paper is available on line at http://www.jbc.org

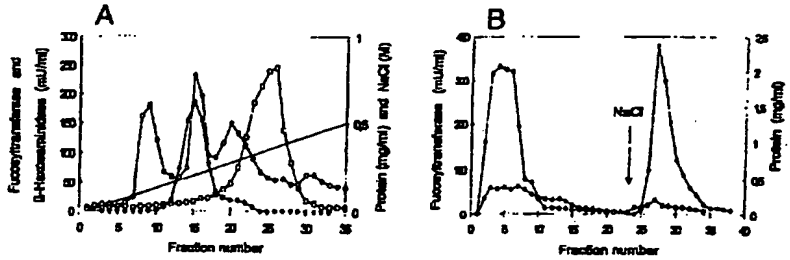

FIG. 2. **Selected steps in the purification of core α1,3-fucosyltransferase.** *A*, separation on S-Sepharose; *B*, separation on GnGn-Sepharose. Details are given under "Experimental Procedures." Fractions from each column were assayed for protein (○), Fuc-T C3 (●), and *N*-acetyl-β-glucosaminidase (□).

Step 3: following dialysis of the eluate from step 2 against buffer B (25 mM sodium citrate buffer, pH 5.3, containing 0.1% Triton X-100 and 0.02% NaN_3) it was loaded onto a column (1.5 × 18 cm) of S-Sepharose equilibrated with the same buffer. Bound protein was eluted with a linear gradient from 0 to 0.5 M NaCl in buffer B. Fractions containing Fuc-T C3 were pooled and dialyzed against buffer C (25 mM Tris-HCl buffer, pH 7.3, containing 5 mM $MnCl_2$ and 0.02% NaN_3).

Step 4: the dialyzed sample was applied to a column (0.5 × 4.5 cm) of GnGn-Sepharose previously equilibrated with buffer C. Elution of the bound protein was accomplished with buffer C containing 1 M NaCl instead of $MnCl_2$.

Step 5: the enzyme was then dialyzed against buffer D (25 mM Tris-HCl, pH 7.3, containing 10 mM $MgCl_2$, 0.1 M NaCl, and 0.02% NaN_3) and subsequently loaded onto a column (0.5 × 4.5 cm) of GDP-hexanolamine-Sepharose. After washing the column with buffer D, Fuc-T C3 was eluted by substituting $MgCl_2$ and NaCl with 0.5 mM GDP. Active fractions were pooled, dialyzed against 20 mM Tris-HCl buffer of pH 7.3, and lyophilized.

Electrophoretic Methods - SDS-PAGE was performed in a Bio-Rad Mini Protean Cell on gels containing 12.5% acrylamide and 1% bisacrylamide. Gels were either stained with Coomassie Brilliant Blue R-250 or silver. Isoelectric focusing of Fuc-T C3 was carried out on precast gels with a pI range from 6 to 9 (Servalyt precotes 6 - 9, Serva) and gels were silver stained according to the manufacturers instructions. For two-dimensional electrophoresis, lanes from the focusing gel were excised, treated with *S*-alkylation reagents and SDS, and subject to SDS-PAGE as described previously (30).

Amino Acid Sequencing and Mass Spectrometric Peptide Mapping — Protein bands were excised from Coomassie-stained SDS-polyacrylamide gels, carboxyamidomethylated, and digested with sequencing grade trypsin according to the in-gel digestion procedure described previously (31). The tryptic peptides were separated by reverse phase HPLC on a 1.0 × 250-mm Vydac C18 at 40 °C with a flow rate of 0.05 ml/min using a HP 1100 apparatus (Hewlett-Packard). Isolated peptides were sequenced with a Hewlett-Packard G1005A protein sequencing system according to the manufacturer's protocol. In addition, the peptide mixture obtained by in-gel digestion was analyzed by MALDI-TOF MS (see below).

Reverse Transcriptase-PCR and cDNA Cloning of Core α1,3-Fucosyltransferase— Total RNA was isolated from 3-day-old mung bean hypocotyls using the SV Total RNA Isolation System from Promega according to the supplier's instructions. To achieve first strand cDNA synthesis, total RNA was incubated for 1 h at 48 °C with avian myeloblastosis virus reverse transcriptase and oligo(dT) primer using the Reverse Transcription System (Promega). First strand cDNA was subjected to PCR using as the sense primer 5′-GCIGARTAYTAYGCIGARAAYAAYATHGC-3′ (S1) and as the antisense primer 5′-CRTADATRTGRTAIACIGTYTC-3′ (S2) or 5′-TADATISWYTCCATYTCRAA-3′ (S3), where I stands for inosin; R for G + A; Y for T + C; H for T + C + A; D for T + G + A; S for G + C; and W for A + T. PCR was performed on 10 μl of the reverse transcriptase reaction in a volume of 50 μl containing 0.1 μmol of each primer, 0.1 mM dNTPs, 2 mM $MgCl_2$, 10 mM Tris-HCl buffer of pH 9.0, 50 mM KCl, and 0.1% Triton X-100. After an initial denaturation step at 95 °C for 2 min, 40 cycles of 1 min at 95 °C, 1 min at 49 °C, and 2 min at 72 °C were run. The final extension step at 72 °C was carried out for 8 min. PCR products were subcloned into pCR2.1 vector using the TA Cloning Kit (Invitrogen) and sequenced.

On the basis of the sequence of PCR product(s), the missing 5′ and 3′ regions of the cDNA coding for Fuc-T C3 were obtained by 5′- and 3′-rapid amplification of cDNA ends (RACE) using the RACE kit from Life Technologies, Inc. according to the manufacturer's recommendations. 3′-RACE was performed with hemi-nested PCR using as antisense primer the universal amplification primer supplied with the kit and as sense primers at first 5′-CTGGAACTGTCCCTGTGGTT-3′ and then 5′-AGTGCACTAGAGGGCCAGAA-3′. Likewise, 5′-RACE was performed by means of hemi-nested PCR using as the sense primer the abridged anchor primer supplied with the kit and as antisense primers either 5′-GAATGCAAAGACGGCACGATGAAT-3′ and then 5′-TTCGAGCACCACAATTGGAAAT-3′ or PCR was performed with an annealing temperature of 55 °C under conditions otherwise as described above. Both 5′- and 3′-RACE products were subcloned into pCR2.1 vector and sequenced.

PCR with Genomic DNA—Genomic DNA was prepared out of lyophilized mung bean hypocotyls by means of the DNeasy Plant Kit (Qiagen) following the manufacturer's instructions. PCR was performed on 200 ng of DNA in 50 μl of solution containing 20 nmol each of fucosyltransferase-specific primers (see below) essentially as described above except that the annealing temperature was raised to 58 °C. The three resulting PCR products (FSP34 59, FSP37–515, and FSP 32–511) were subcloned into pCR2.1 vector using the TA cloning Kit (Invitrogen) and sequenced. Forward primers 5′-GGAACCATCCACCCATAAC-3′, 5′-AGTCGTGTTCGGTTGGATGT-3′, and 5′-CTGGAACTGTCCCTGTGGTT-3′ and reverse primers 5′-CTCAGCATAGTATTCTGCTG-3′, 5′-GAAGGAGCAAAGTCCTGAATA-3′, and 5′-GTACCATTTAGCGCAT-3′ were used to cover cDNA regions from 174 to 522, 392 to 944, and 890 to 1550 bp, respectively.

DNA Sequence Analysis Sequences of subcloned fragments were determined by the dideoxynucleotide chain termination method using an ABI PRISM Dye Terminator Cycle Sequencing Ready reaction Kit and an ABI PRISM 310 Genetic analyzer (Perkin-Elmer). T7 and M13 forward primers were used for sequencing the PCR products cloned in pCR2.1. Sequencing of both strands of the complete coding region was performed by the Vienna VBC Genomics-Sequencing Service using the cycle sequencing method with infrared labeled primers (IRD700 and IRD800) and a LI-COR Long Read IR 4200 sequencer (Lincoln, NE).

Expression of Recombinant Fuc-T C3 in Insect Cells The coding region of the putative Fuc-T C3 cDNA including the cytoplasmic and the transmembrane regions was amplified using the forward primer 5′-cggcggatcCGCAATTGAATGATG-3′ and the reversal primer 5′-ccggctgcaGTACCATTTAGCGCAT-3′ by means of the Expand High Fidelity PCR System (Roche Molecular Biochemicals). The PCR product was double digested with *Pst*I and *Bam*HI and subcloned into alkaline phosphatase-treated baculovirus transfer vector pVL1393 previously double digested with *Pst*I and *Bam*HI. To allow homologous recombination, the transfer vector was co-transfected with BaculoGold viral DNA (PharMingen, San Diego, CA) into Sf9 insect cells in IPL-41 medium containing Lipofectin. After 5 days of incubation at 27 °C, various volumes of supernatant containing recombinant virus were used for infection of Sf21 insect cells. After incubation for 4 days at 27 °C in IPL-41 medium containing 5% fetal calf serum, the Sf21 cells were harvested and washed twice with phosphate-buffered saline. The cells were resuspended in 25 mM Tris-HCl buffer of pH 7.4 containing 2% Triton X-100 and disrupted by sonication on ice. This homogenate as well as the culture supernatant were assayed for Fuc-T C3 activity. Mock infections were performed with recombinant baculovirus encoding tobacco GlcNAc transferase I (28).

Analysis of the Transferase Product Dabsylated GnGn-hexapeptide (2 nmol) was incubated with insect cell homogenate containing recombinant Fuc-T C3 (0.08 milliunit) in the presence of non-radioactive GDP-L-fucose (10 nmol) under conditions otherwise identical to those described for determination of transferase activity (see above). A control experiment was performed with homogenate from mock-infected insect

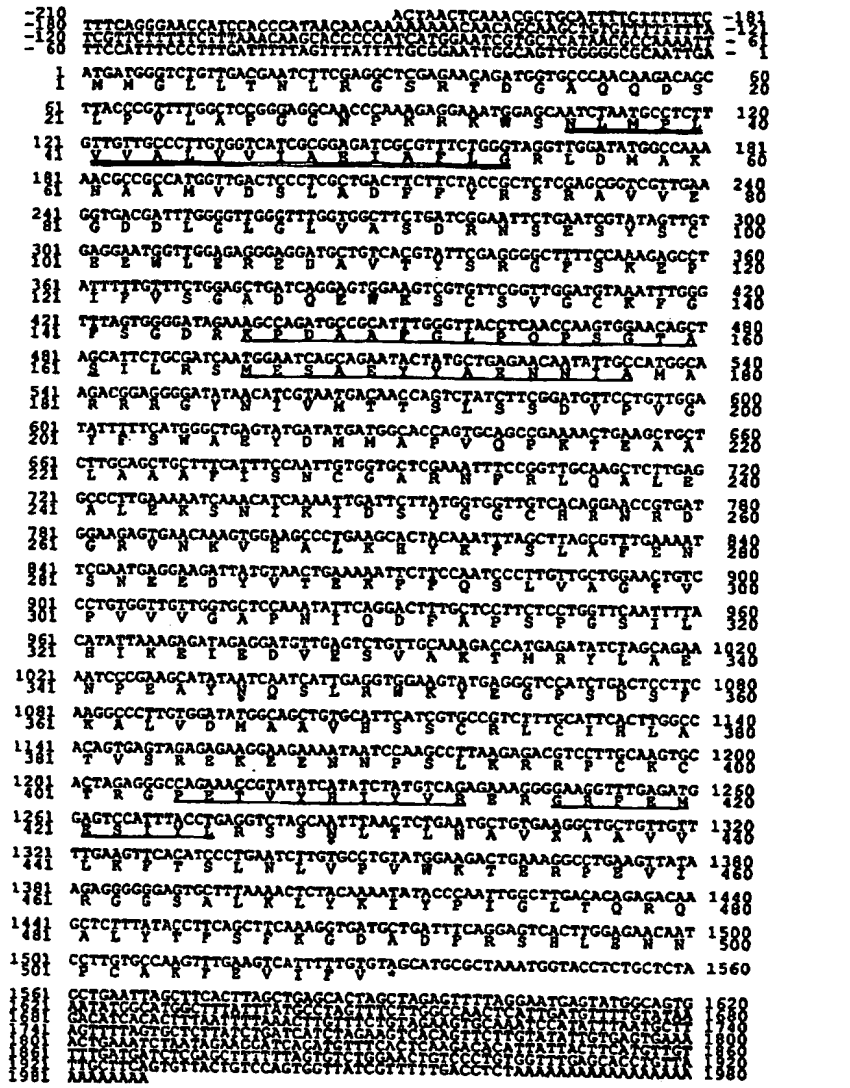

FIG. 4. **cDNA and deduced amino acid sequence of mung bean core α1,3-fucosyltransferase.** The complete cDNA comprises 2198 base pairs including an open reading of 1530 base pairs which encodes a 510-amino acid protein with a theoretical molecular mass of 56.8 kDa. The hydrophobic putative transmembrane domain is *double underlined*. The peptide sequences obtained by amino acid sequencing are indicated by *single underlining*. Consensus sites for asparagine-linked glycosylation are indicated by *diamonds*.

Due to the lack of information about the natural N terminus of Fuc-T C3, the possible N terminus can only be deduced from potential initiation codons between the putative transmembrane region (see below) and the first stop codon toward the 5′ end. The open reading frame that starts with the first Met residue located right beneath a stop codon encodes a protein of 510 amino acids, a molecular mass of 56.8 kDa, and a calculated pI of 7.51 (Fig. 4). A theoretical tryptic peptide map of the deduced complete amino acid sequence exhibited significant similarity to the map of purified Fuc-T C3 (data not shown).

Expression and Characterization of Recombinant Fuc-T C3—The coding region of Fuc-T C3 was engineered into a baculovirus transfer vector. Various amounts of progeny virus from the co-transfection were used to infect Sf21 insect cells. In the best batch obtained, total fucosyltransferase activity of cells and supernatant was about 30 times higher than in the mock infected control batch. The endogeneous activity measured in the absence of recombinant transferase arises, however, essentially from insect Fuc-T C6 and only to a marginal extent from Fuc-T C3 (32, 36). Thus, the increase in Fuc-T C3 caused by the recombinant baculovirus is well above 100-fold.

Similar to the natural enzyme, the recombinant transferase displayed a broad maximum of activity around pH 7.0 when measured in Mes-HCl buffer and the presence of divalent cations, in particular of Mn^{2+}, enhanced its activity. Among the acceptors employed, GnGn-peptide gave the highest incorporation rates under standard assay conditions, closely followed by GnGnF^6-peptide and M5Gn-Asn (Table II). The apparent K_m values for the acceptor substrates GnGn-peptide, GnGnF^6-peptide, M5Gn-Asn, and for the donor substrate GDP-fucose were estimated to be 0.19, 0.13, 0.23, and 0.11 mM, respectively. No transfer was observed to MM-peptide which lacks the terminal GlcNAc residue on the 3-linked mannose regarded to be a structural requirement for core fucosyltransferases (1, 2, 37). By the standard assay, fucosyl transfer to GalGal-peptide could not be observed. However, a low rate of incorporation was demonstrated by MALDI-TOF MS (see later). Recombinant Fuc-T C3 was inactive toward common acceptors used for the determination of blood group α1,3/4-fucosyltransferases which transfer fucose to GlcNAc residues at the nonreducing termini

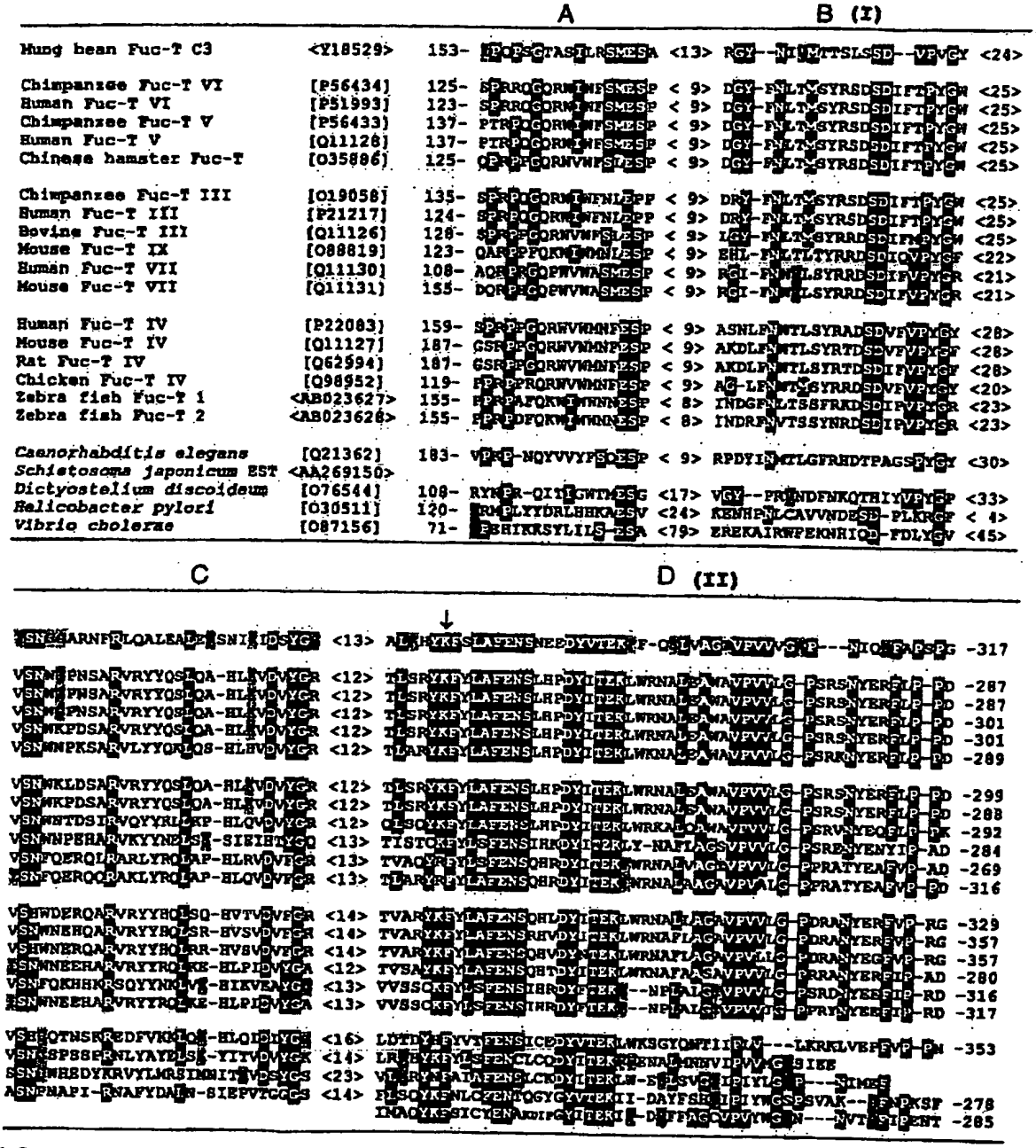

FIG. 6. **Conserved regions of core and blood group α1,3-fucosyltransferases.** Four regions of apparent homology between mung bean Fuc-T C3 and most currently known α1,3/4-fucosyltransferases are shown. Conserved residues are represented by *white letters on black background* or, if common to only a few transferases, on *gray background*. The blocks B and D represent the highly conserved regions I and II previously described (43, 44). In the case of the putative fucosyltransferase from *Dictyostelium discoideum* and the EST from *S. japonicum*, the gene products have not yet been analyzed. A lysine residue shown to be essential for activity of human Fuc-T V and VII is marked by an *arrow* (45). The number of residues between the depicted partial sequences are given in *brackets*. Transferases are identified by SwissProt (*square brackets*) or, if not applicable, by GenBank accession numbers.

of the reducing terminal GlcNAc (Fig. 7) (4, 36, 40). No other known structural feature of *N*-linked oligosaccharides exerts such a strong and characteristic effect on the retention time of pyridylamino glycans (40). The compounds mass of 1564.5 agreed exactly with the mass expected for the sodium adduct of the pyridylamino glycan. To exclude any possibility of fucose being linked to a nonreducing terminal GlcNAc and to allow comparison of retention time with a reference oligosaccharide analyzed previously by independent methods, *i.e.* pyridylaminated MMF^3 from honeybee venom phospholipase (Fig. 1) (26), the product was digested with *N*-acetyl-β-glucosaminidase. Indeed, the putative MMF^3 coeluted with the reference glycan.

matically between mung bean seedlings from two suppliers.

Based on partial peptide sequences, a cDNA putatively encoding Fuc-T C3 was cloned. A first confirmation of the authenticity of this cDNA came from comparison of the theoretical tryptic map of the translated sequence with the peptide masses obtained from purified Fuc-T C3. Unfortunately, no matching peptides could be found for a large portion at the N terminus comprising the putative transmembrane and cytoplasmic domain. While suppression effects are not uncommon in MALDI-MS, this might also indicate that the purified enzyme was a truncated form lacking these domains. Expression of the cloned cDNA in the baculovirus-insect cell system finally confirmed that it encoded Fuc-T C3. The recombinant transferase used the acceptors GnGn-, M5Gn-, and the "mammalian" GnGnF[6]-peptide with similar efficiency. It is, to our knowledge, the first time that the biosynthetic intermediate M5Gn is shown to be a potential acceptor for fucose. Remarkably, GnGnF[6] which certainly does not occur in plants, appeared to be the best acceptor in kinetic terms. A β1,4-linked Gal residue on the 3-arm inhibits the action of Fuc-T C3. This explains the reduction of fucosylated glycans in plant cells expressing recombinant β1,4-galactosyltransferase (42). Incorporation of fucose by the recombinant enzyme rendered a glycopeptide resistant against *N*-glycosidase F which is in keeping with the inability of this glycosidase to act on core α1,3-fucosylated substrates (27). In addition, the product was analyzed by reverse phase HPLC using the authentic reference glycan MMF[3] from honeybee venom phospholipase A_2.

Considering the evolutionary distance between Fuc-T C3 and mammalian Lewis blood group α1,3/4-fucosyltransferases and their different acceptor substrates, we did not expect to find sequence homologies to this enzyme family. Indeed, the amino acid sequence of Fuc-T C3 displays an insignificant overall homology of 18–21% when compared with these fucosyltransferases. However, a large part of exon II (residues 154 to 350) exhibits, *e.g.* 31% identical residues with chimpanzee Fuc-T VI. The conserved residues are found clustered in four regions as depicted in Fig. 6. Two of these clusters constitute the highly conserved regions identified previously by Breton *et al.* (42). Especially, region D (region II in Refs. 43 and 44) appears to be highly conserved between mammalian Lewis and plant core fucosyltransferases. This region also contains a Lys residue identified to be essential for acitivity of human Fuc-T V and Fuc-T VII (identified by an *arrow* in Fig. 6) (45). Mammalian Fuc-T contain in region B (region I in Refs. 43 and 44) a DSD-motif suggested to be part of the catalytic site (46). In mung bean Fuc-T C3, a SSD-motif is found at this site. Remarkably, bacterial and protozoan fucosyltransferases exhibit a lower degree of homology than most mammalian Fuc-Ts with the possible exception of a putative *Schistosoma japonicum* Fuc-T of which only an EST exists (44). Although exon III on its own is not significantly homologous to the Lewis blood group α1,3/4-fucosyltransferases, when coupled to exon II, its first part (residues 351 to 384) can be tentatively aligned with, *e.g.* human Fuc-T VI or V to reveal the conserved motifs Arg-Trp-(Arg/Lys) (with Trp-Arg being found in all mammalian Fuc-Ts) and Cys-*X*-*Y*-Cys, where *X* very often is a basic residue. In contrast, a Cys residue which is located between the conserved regions A and B and which has been shown to be involved in binding of GDP-fucose by human Fuc-T III, V, and VI (47), is not seen in Fuc-T C3. In other animal fucosyltransferases, this Cys residue is replaced by Ser or Thr. However, the only hydroxy amino acid found at this site of Fuc-T C3 is Tyr. It shall be noted, that no sequence similarities of residues 385–510 from mung bean Fuc-T C3 with animal α1,3/4-fucosyltransferases are to be expected because these much shorter enzymes do not contain a comparable region. Despite a similar substrate specificity, the recently cloned porcine and human core α1,6-fucosyltransferases do not exhibit any obvious sequence similarities with mung bean core α1,3-fucosyltransferases (21, 22).

Sequencing of the genomic region containing the open reading frame of Fuc-T C3 predicted three introns dispersed between four exons. Exon II with its conserved regions and the cytoplasmatic region on exon I are separated by a large intron of 771 bp. Introns interrupting the coding region have also been found in mouse FTVII (Q11131) (48) and in *Caenorhabditis elegans* CEFT-1 (Q21362) (49), the latter containing nine introns. In contrast, in many other α1,3-fucosyltransferases the entire coding sequence is contained within a single exon (44, 50, 51).

Mung bean Fuc-T C3 is the first plant fucosyltransferase and the first core α1,3-fucosyltransferase which has been cloned and sequenced. Our designated abbreviation "Fuc-T C3" takes into account the transfer of fucose into the 3 position of the core GlcNAc. Following the nomenclature of other fucosyltransferases, the enzyme may also be designated Fuc-T X. More significantly, it will now be possible to express large quantities of Fuc-T C3, thus enabling the *in vitro* synthesis of a variety of core α1,3-fucosylated *N*-glycans or *N*-glycopeptides from acceptors which are derived from mammalian glycoproteins. These "vegetabilized" structures will aid in the further elucidation of the role of core α1,3-fucose in the immunogenicity of plant and insect glycoproteins.

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EXHIBIT D

Declaration of Dr. Iain Wilson

I, Iain Benedict Howe Wilson, D.Phil., hereby declare as follows:

1. I studied biochemistry at the University of Edinburgh and the University of Oxford and graduated as a doctor of philosophy in 1991.

2. Since 1999 I am working at the University of Agriculture, Vienna, and am since March 2002 associate professor at the Institute of Chemistry.

3. My curriculum vitae is attached hereto as appendix A.

4. I am the author/co-author of over 30 publications whereby these publications relate to the technical field of glycosylation of proteins and glycosyltransferases. A list of these publications is attached hereto as appendix B.

5. I have read the present application EP 1 151 109 A1 as well as the Office Action according to article 96(2) EPC dated 13 January 2003.

6. I do not agree with the assertions in the Office Action item 5.1 that claim 1 of the present application relates by the term "50% homology to sequence SEQ ID No. 1" to a too high number of possible DNA molecules so that the person skilled in the art would not be able to detect and isolate the inventive DNA molecules without undue burden.

7. With the help of programs, such as BLAST at the EMBL or SWISSPROT internet sites, it is possible to define the exact percentage of homology or identity between two given sequences. This is a very common method and since it is carried out electronically the result can be expected immediately. Furthermore, since these programs are accessible on internet this method can be carried out from any computer or processor without the need of additional equipment.

8. Various methods are known of how to find sequences which could show at least 50% homology to SEQ ID No. 1 and which therefore potentially fall within the scope of protection of claim 1 of the present application.

One possibility is defined in claim 24 of the present application whereby a known DNA molecule, for example the inventive DNA molecule according to claim 1 of the present application, which codes for a GlcNAc-α1,3-fucosyltransferase is

added to a sample and sequences which bind to this added DNA molecule can be tested for homology as mentioned above.

Another possibility would be to check data bases for published sequences which potentially fall into the scope of protection of claim 1 of the present application and to check these with respect to homology according to the method as mentioned above.

9. These are only examples of how potential DNA molecules can be provided with which the homology test as mentioned above can be carried out. These methods (among others) belong to basic techniques which can be carried out by any person with basic laboratory experience in this technical field. Therefore, the person skilled in the art will be able to isolate or detect a DNA molecule (on the basis of the knowledge of SEQ ID No 1) and to verify the percentage of homology between the detected DNA molecule and the sequence according to SEQ ID No. 1 without undue burden.

10. A further feature necessary for a DNA molecule to fall under the scope of protection of claim 1 of the present application is that the DNA molecule must code for a protein with fucosyltransferase activity. In order to test this the basic method comprises adding the respective protein to a sample comprising labelled fucose and an acceptor which may be any peptide to be glycosylated. This acceptor may be for example bound to a carrier which will facilitate the detection. After a reaction time the sample is usually washed and a content of bound fucose to the acceptor is measured. The activity of the fucosyltransferase is defined in the present application, page 5, 1st paragraph, as being positive if the activity measurement is higher by at least 10 to 20%, in particular at least 30 to 50%, than the activity measurement of the negative control. Furthermore, it is possible to varify the structure of the glycoprotein additionally for example by means of HPLC. These methods are well known to the person skilled in the art and they are for example described in publications by Staudacher et al., 1998, Anal. Biochem. 246, 96-101; and by Staudacher et al., 1991, Eur. J. Biochem. 199, 745-751. This is also mentioned in the present application on page 5, 1st pragraph.

Such a method is described in more detail on page 5, 2nd paragraph as well as in example 6 on page 30 of the present application.

However, even without these examples for measuring the activity of a potential fucosyltransferase, the person skilled in the art who knows in what way the α1,3-fucosyltransferase acts (e.g. which molecules it adds to which proteins and in

which positions), whereby this information is published as mentioned in the specification of the present application, will be able to set up and design a protocol with which the activity of a given transferase can be tested.

11. Therefore, in order to test whether a given DNA molecule falls under the scope of protection of the present application, one part of the method will be carried out electronically and therefore is certainly not an undue burden and the second part of the method comprises a biochemical activity test which can be carried out even in a high throughput assay which means that a large number of proteins can be tested at once.

12. With the data given in the present application sufficient information is provided to allow a person skilled in the art using his common general knowledge to perform the invention (to decide whether or not a given DNA molecule shows all features according to claim 1 of the present application) without undue burden and without needing inventive skills. Of course, theoretically a large number of potential candidates of DNA molecules show 50% homology to the sequence according to SEQ ID No. 1. However, the activity assay does not constitute undue burden to the person skilled in the art. On the contrary, with the help of high throughput techniques which belong to basic laboratory methods the test with respect to α1,3-fucosyltransferase activity can be carried out quickly and efficiently, e.g. without undue burden.

13. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.

4/4/2003
Date

Iain Wilson, D.Phil.

Resume

Personal information

Surname/First names:	Iain Benedict Howe Wilson
Address:	Department of Chemistry of the University of Natural Resources and Applied Life Science, Vienna 1190 Vienna, Austria
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Date of Birth:	22 August 1967
Gender:	Male

Work experience

Dates:	March 2002 to present
Occupation or position held:	Contract Associate Professor, employed as „extraordinary professor“
Name and address of employer:	Department of Chemistry, University of Natural Resources and Applied Life Science, Vienna
Dates:	January 1999 to February 2002
Occupation or position held:	Substitute Assistant Professor
Name and address of employer:	Working group on Glycobiology Department of Chemistry,University of Natural Resources and Applied Life Science, Vienna
Dates:	May 1998 to December 1998
Occupation or position held:	Postdoctoral Research Assistant
Name and address of employer:	funded by Medical Research Council
Collaborator:	Professor Grahame Hardie, University of Dundee, Scotland
Dates:	May 1997 to May 1998
Occupation or position held:	Postdoctoral Research Assistant
Name and address of employer:	funded by Mizutani Foundation
Collaborator:	Professor Michael Ferguson, University of Dundee, Scotland
Dates:	January 1995 to May 1997
Occupation or position held:	Leverhulme Trust Study-Abroad Studentship
Collaborator:	Professor Friedrich Altmann, University of Natural Resources and Applied Life Science, Vienna

Dates:	January 1992 to December 1994
Occupation or position held:	Postdoctoral Research Assistant
Name and address of employer:	funded by SERC
Collaborator:	Dr. Sabine Flitsch, Dyson Perrins Laboratory, Oxford, UK

Dates:	1989 to 1994 (one term per year)
Occupation or position held:	Tutor, Physical Chemistry for Biochemists Problem Classes
Name and address of employer:	University of Oxford

Dates:	1990 (two tutorials)
Occupation or position held:	Tutor, Glycobiology for Biochemistry Students
Name and address of employer:	Wadham College, Oxford

Dates:	October 1988 to September 1991
Occupation or position held:	Postgraduate Research Student
Name and address of employer:	funded by Medical Research Council
Collaborators:	Dr. Thomas Rademacher and Professor Raymond Dwek, Department of Biochemistry, University of Oxford, UK

Other scientific activities:

Exercises in Biochemistry II (605.028): Enzyme example and literature example

Lecture course:	Biochemistry of complex carbohydrates (605.308, partial) Biochemical analytics (605.046; partial)

Supervisor of doctoral canditates:	Dubravko Rendić (Neose Project) Monika Bencúrova (FWF Project) (partial)

Mini-review editor (Europe), Glycoconjugate Journal

Third-party-funded projects:

Neose Technologies Glycoscience Research Award 1999 to 2002
Phosphorylation of β1,4-galactosyltransferase ($ 150,000.00)

Funds for the Promotion of Scientific Research (FWF) 1999 to 2002
The structural, biosynthetic and genetic basis of anti-horseradish peroxidase epitopes in *Drosophila melanogaster* and *Caenorhabditis elegans* (ATS 2,250,720.00)

Hochschuljubiläumsstiftung of the City of Vienna 1999 to 2000

Carbohydrate mediated immunological cross-reactions of glycoproteins from plants and invertebrates (ATS 50,000.00)

Funds for the Promotion of Scientific Research (FWF) 2002 to 2003

Glycosylation of *Caenorhabditis elegans* (ATS 1,109,218.00)

Administrative activities

Supervision of the Dok-database of the University of Natural Resources and Applied Life Science, Vienna for the whole Department of Chemistry

Elaboration of statistics and work reports for the University Board of Trustees

Organisation and administration (accounting included) of the above-mentioned third-party-funded projects

Education and Training

Dates:	2001
Title of qualification awarded:	Qualifying examination for lecturing at a university in the field of „Biochemistry"
Name and type of organisation:	University Natural Resources and Applied Life Science, Vienna
Dates:	1998 to 1991
Title of qualification awarded:	Dphil, Biochemistry
Thesis entitled:	Studies on galactosyltransferase in the pathology of rheumatoid arthritis
Name and type of organisation:	University of Oxford
Dates:	1991
Title of qualification awarded:	Cchem, MRSC
Name and type of organisation:	Royal Society of Chemistry
Dates:	1984 to 1988
Title of qualification awarded:	Bsc (Hons, first class), Biochemistry
Project:	Project on cloning of *Onchocerca* phosphofructokinase cDNA)
Name and type of organisation:	University of Edinburgh

IAIN B. H. WILSON Publication List

Monograph

Bill, R.M., Revers, L. and Wilson, I.B.H. (1998) Protein Glycosylation, Kluwer Academic Publishers.

Papers/Reviews

Goodhew, C.F., Wilson, I.B.H., Hunter, D.J.B. and Pettigrew, G.W. (1990) The cellular location and specificity of bacterial cytochrome c peroxidases. *Biochem. J.* 271, 707-712.

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Wilson, I.B.H.* and Hardy, B.J. (1996) Glycoscience and the Internet. *Trends in Glycoscience and Glycotechnology* 8, 301-310. (Review)

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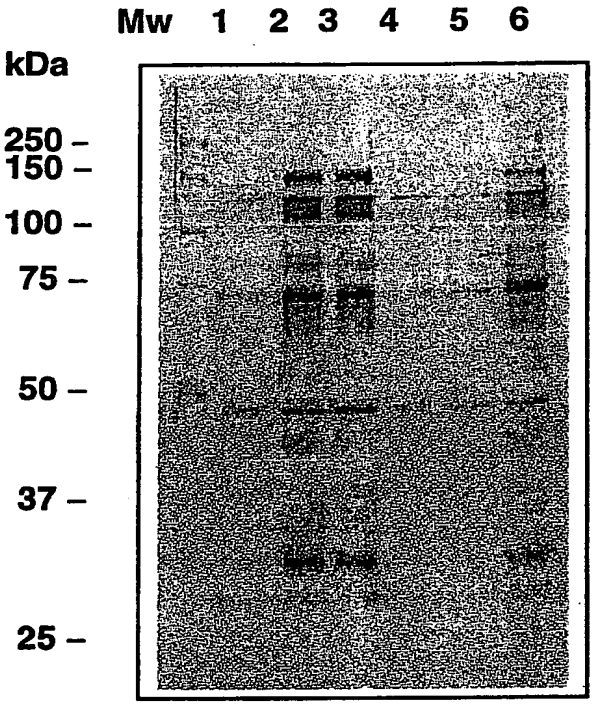
EXHIBIT E

EXAMPLE

Suppression of formation of antigenic N-glycan in Drosophila

A fruitfly (*Drosophila melanogaster*) homologue FucTA of the mung bean core α1,3-fucosyltransferase (Leiter et al., 1999; Document D1) has been recently identified, cloned and it has been found to be enzymatically active. More recently it was shown by *in situ* hybridisation *Drosophila* core α1,3-FucTA mRNA to be expressed in the fly embryonic central nervous system. The discovery of rapid gene disruption by introduction of homologous double stranded (ds) RNA fragments, also called RNAi effect, has accelerated our attempts on the function elucidation of the fly core α1,3-fucosyltransferase FucTA. Disruption of the fly FucTA mRNA in the cultured *Drosophila* neuronal cells by homologous dsRNAs has led to the loss of carbohydrate anti-HRP epitope, known as a neuronal marker (s. Figure C). These data clearly suggest the FucTA *in vivo* to be involved in the biosynthesis of the neuronal carbohydrate anti-HRP epitope.

To exemplify the importance of α1,3-fucosyltranferases in vertebrates, however acting terminally, mice lacking the α1,3-fucosyltransferase FucTVII exhibit a leukocyte adhesion deficiency by impairing the fucosylation of leukocyte receptors.



RNAi induced with fragments of:

1: core α1,3–FucTA
2: core α1,6–FucT
3: *Drosophila* lectin CG2958
4: core α1,3–FucTA + core α1,6–FucT
5: core α1,3–FucTA + CG2958
6: Nervana Nrv 2.2

Fig. C: Drosophila neuronal cells were incubated with the individual dsRNA fragments and incubated for four days at 26°C 1x106 cells after RNAi was loaded per lane and assayed on a Western blot with anti–HRP antibodies. Notice that only cells treated with the core α1,3 FucTA derived dsRNAs caused the loss of neural carbohydrate anti–HRP epitope (lanes 1,4,5).

EXAMPLE

Knock out of core 1,3-fucosyltransferase in the moss *Physcomitrella* patens

Description

Different technologies exist for the downregulation of enzyme activity on a molecular level. Using antisense, ribozyme and RNAi methods downregulation only up to 95% could be reached. In contrast, the knock out of a gene results in complete loss of the corresponding protein and its activity as well. Knock out of a gene could be achieved by homologous recombination via disruption or replacement of the endogenous gene. One of the few organisms in which efficient homologous recombination occurs is the moss *Physcomitrella patens*. Downregulation of genes would be very important especially for the production of recombinant proteins in plants. For example, there are two additional sugar residues (xylosyl and fucosyl residues) in the core structure of N-glycans on glycoproteins of plant-derived compared to mammalian derived N-glycans. These residues are considered to be allergenic to humans.

In *Physcomitrella patens* the GDP-L-Fuc:Asn-linked GlcNAc α1,3-fucosyltransferase (Accession CAD22109) showing 57% identity at the amino acid level to mung bean α1,3-fucosyltransferase described by Leiter et al. 1999 (Document D1) could be knocked out by homologous recombination. The knock out of core α1,3-fucosyltransferase resulted in the complete loss of α1,3-linked fucosyl residues on the core structure of N-glycans isolated from *Physcomitrella patens* and analysed by MALDI-TOF mass spectrometry (table A).

Methods

The fucosyltransferase knock out in *Physcomitrella patens* was performed by cloning a selection cassette (npt II gene under control of the 35 S promotor and the 35 S termination signal)

between homologous sequences (5' flanking region 715 bp; 3' flanking region 812 bp) corresponding to the fucosyltransferase gene of *Physcomitrella patens*. The vector pCR3 was used for cloning resulting in the new vector named pCR3 fucosyltransferase ko/npt II ki. For each transformation 20 microgramm of this plasmid were digested with two restriction enzymes resulting in two linearised fragments. One fragment contained the selection cassette flanked by the homologous sequences for the Pp fucosyltransferase the other fragment contained the vector sequences.

Transformation and regneration of *Physcomitrella patens* protoplasts were performed under standard conditions. For selection, Knop's media were supplemented with 50 mg/l G418.

To analyse positive plants, the homologous recombination was checked by PCR using specific primer combinations. The plants positive for homologous recombination were tested in RT-PCR experiments which confirmed the results of the PCR.

Three knock out plants were used for analysing the N-glycan structures. Compared to wild type plants the knock out lines showed normal growth and differentation.

N-glycans were analysed by MALDI-TOF mass spectrometry (table A). α1,3-linked fucosyl residues were completely lost confirming the knock out of the core α1,3-fucosyltransferase gene in *Physcomitrella patens*.

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Table A

		MOSS Knock--out	
M+ Na	M + K	Structures normal	Structures in knock out plants FucT2.3, FucT2.5. FucT2.8
933,8	949,8	Man3 (MM)	Man3 (MM)
1065,7	1081,7	MMX	MMX
1080.0	1096.0	MMF	
1096.0	1112,0	Man4	Man4
1137,0	1153.0	MGn /GnM	MGn /GnM
1212,1	1228,1	MMXF	
1227,8	1243,8	Man4X	
1258,4	1274,4	Man5	Man5
1269,1	1285,1	GnMX / MGnX	GnMX / MGnX
1299,2	1315,2	Man4Gn	Man4Gn
1340,2	1356,2	GnGn	GnGn
1415,5	1431,5	GnMXF / MGnXF	
1420,2	1436,2	Man6	Man6
1431,4	1447,4	Man4GnX	Man4GnX
1445,3	1461,3	Man4GnF	
1472,1	1488,1	GnGnX	GnGnX
1486,4	1502,4	GnGnF	
1577,4	1593,4	LMXF / MLXF / Man4GnXF	
1582,4	1598,4	Man7	Man7
1618,5	1634,5	GnGnXF	
1739,5	1755,5	Man5GnXF	
1744,5	1760,5	Man8	Man8
1780,4	1796,4	LGnXF / GnLXF	(LF)GnX / Gn(LF)X
1907,1	1923,1	Man9	Man9
1926,7	1942,7	(LF)GnXF / Gn(LF)XF	
2068,8	2084,8	Man9Glc1	Man9Glc1
2088,9	2104,9	(LF)LXF / L(LF)XF	(LF)(LF)X
2235,0	2251,0	(LF)(LF)XF	

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